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Stage specific reprogramming of mouse embryo liver cells to a beta cell-like phenotype

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ABSTRACT

We show that cultures of mouse embryo liver generate insulin-positive cells when transduced with an adenoviral vector encoding the three genes: *Pdx1*, *Ngn3* and *MafA* (*Ad-PNM*). Only a proportion of transduced cells become insulin-positive and the highest yield occurs in the period E14–16, declining at later stages. Insulin-positive cells do not divide further although they can persist for several weeks. RT-PCR analysis of their gene expression shows the upregulation of a whole battery of genes characteristic of beta cells including upregulation of the endogenous counterparts of the input genes. Other features, including a relatively low insulin content, the expression of genes for other pancreatic hormones, and the fact that insulin secretion is not glucose-sensitive, indicate that the insulin-positive cells remain immature. The origin of the insulin-positive cells is established both by co-immunostaining for α-fetoprotein and albumin, and by lineage tracing for *Sox9*, which is expressed in the ductal plate cells giving rise to biliary epithelium. This shows that the majority of insulin-positive cells arise from hepatoblasts with a minority from the ductal plate cells.

1. Introduction

During embryonic development cells undergo a sequence of developmental decisions, usually in response to external signals (Gilbert, 2010; Slack, 2012; Wolpert and Tickle, 2011). At each stage the cell state is defined by a combination of transcription factors that controls the activity of the appropriate repertoire of genes. For each cell lineage the end product of the sequence of decisions is a specific differentiated cell type. It is possible to alter the pathway taken by a lineage in two ways. During the stage of competence to extracellular signals, the cell may be caused to develop down a different pathway by exposure to a different extracellular factor or to a different concentration of the same factor. This is why grafts of embryonic tissue from one position to another are often caused to develop in a manner consistent with their new position. In addition it is possible to alter the course of development by misexpression of those transcription factors which define the intermediate or terminal states of cell differentiation.

To some extent it has even proved possible to reprogram terminally differentiated cells by misexpression of transcription factors appropriate to a different cell type (Gurdon and Melton, 2008; Zhou and Melton, 2008). However such procedures frequently have no effect or give partial transformations resulting in mixed phenotypes. Because of the importance of the pancreatic beta cell for control of blood sugar, and its role in diabetes, considerable effort has gone into elucidation of the pathways of pancreatic development in recent years (Gittes, 2009; Grapin-Botton et al., 2007; McKnight

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et al., 2010; Murtaugh and Melton, 2003; Rieck et al., 2012). Methods for generating cells with a beta-like phenotype have also attracted much attention because of the prospect that these cells could be used for transplantation therapy of type 1 diabetes (Bonner-Weir and Weir, 2005; Gangaram-Panday et al., 2007; Miszta-Lane et al., 2006).

In 2008 Zhou et al. showed that the three gene combination *Pdx1*, *Ngn3* and *MafA* could reprogram pancreatic exocrine cells of adult mice into a beta-like phenotype (Zhou et al., 2008). PDX1 normally controls growth and development of the pancreatic bud, NGN3 is required for formation of endocrine progenitors, and MAFA (and also PDX1 again) are required for maturation of beta cells (Gittes, 2009; Grapin-Botton et al., 2007; McKnight et al., 2010; Murtaugh and Melton, 2003; Rieck et al., 2012). Our laboratory had previously found that overexpression of the gene for an activated form of PDX1 (PDX1-VP16) in the immature liver of *Xenopus* tadpoles could bring about a reprogramming to pancreas (Horb et al., 2003). In view of the potency of *Pdx1* + *Ngn3* + *MafA* (here referred to as PNM) on adult pancreatic exocrine cells, we were interested to find what was the susceptibility of the cells of the liver to this gene combination. The liver is developmentally related to the pancreas as it arises from the same region of the embryonic endoderm, which becomes partitioned into liver and pancreatic buds depending on the concentration of FGF and BMP secreted by the neighboring cardiac mesoderm (Chung et al., 2008; Deutsch et al., 2001; Gouon-Evans et al., 2006; Zaret and Grompe, 2008). This close developmental relationship may mean that the chromatin configuration of mature liver cells still allows access by pancreatic transcription factors to their target genes and so their overexpression can be effective at phenotypic reprogramming (Kraus and Grapin-Botton, 2012). In studies on adult mice we have found that PNM has two effects. It will reprogram hepatocytes to a mixed phenotype which has some properties of beta cells and some of hepatocytes. It will also reprogram a *Sox9* positive cell population, probably cells of small bile ducts, to a different mixed phenotype having some properties of beta cells and some of ducts (Banga et al., 2012). In view of the previous experience with *Pdx1-VP16* in *Xenopus*, we were interested to examine the susceptibility of embryonic liver to this three gene combination and this is done in the present paper.

The experiments were carried out using in vitro cultures of cells from embryonic mouse liver buds. These are mixed cultures containing both epithelial and mesenchymal cells and, later on, also some ductal plate cells. Ductal plate cells express *Sox9* and later give rise to the epithelium of the biliary system (Antoniou et al., 2009; Carpentier et al., 2011; Delous et al., 2012; Lemaigre, 2003). Cultures were transduced with *Ad-PNM*, which is an adenoviral vector encoding all three genes: *Pdx1*, *Ngn3* and *MafA*. Adenovirus is a DNA virus which is suitable for transient overexpression as it does not integrate into the host cell genome.

Our results show that PNM brings about a partial reprogramming to insulin-positive cells and that the competence to respond declines during the second half of the gestation period. The cells of origin are mostly hepatoblasts although there is also a small contribution from *Sox9*-positive cells, which are presumed biliary precursors. As seen previously with pancreatic exocrine cells (Akinci et al., 2012), the reprogramming appears to be incomplete. Although the cells do secrete insulin they do not respond appreciably to glucose-stimulation, as do normal beta cells. They also have lower insulin content than normal beta cells and express hormones characteristic of other pancreatic endocrine cell types. These are all characteristics of immature beta cells so suggest that the embryonic hepatoblasts are diverted in their development by the three transcription factors to become immature pancreatic endocrine cells.

2. Results

2.1. *Induction of a partial beta cell phenotype in embryonic liver cultures*

Embryonic liver cultures were established from CD1 mice and were transduced with *Ad-PNM*. Previous work with this three gene vector has shown that it is much more effective than co-transfection with individual vectors encoding the three genes separately (Akinci et al., 2012). The cultures were set up from gestation days E12–E18. Liver buds were dissected from the embryos, minced with forceps and collagenased to dissociate them into single cells. These were filtered through a 100 micron mesh and plated in Kubota's medium (Kubota and Reid, 2000) on a Matrigel substrate. Cultures were exposed to *Ad-PNM* for three days and on the following day were fixed for immunostaining or processed for Q-RT-PCR analysis.

The appearance of control cultures is shown in Fig. 1. They appear as islands of epithelial cells separated by areas of mesenchyme. The epithelial cells stain positive for a number of hepatoblast or hepatocyte markers: α-fetoprotein (AFP), E-cadherin, epithelial cell adhesion molecule (EpCAM), OV6, and albumin. The AFP level decreases and the albumin level increases over the period E12–E18. Following *Ad-PNM* transduction, a large number of insulin-immunopositive cells appear (Fig. 2), while none are seen in control cultures. Fig. 2A–C shows the concordance between insulin expression and the expression of the three virus-encoded proteins: PDX1, NGN3 and MAFA. Many more cells become transduced with virus than express insulin. Those that do express insulin are not those showing the highest level of virus-encoded proteins, rather they appear to show medium levels. There was a pronounced difference in the number of insulin-positive cells seen depending on the embryonic stage at which the cultures had been initiated. The overall proportion of insulin-positive cells peaked at about 17% for cultures initiated in the period E14–16 (Fig. 3A–D). In Fig. 4 are presented quantitative data. This shows that the level of virus transduction, measured by the number of cells expressing PDX1, increased only slightly with stage, (E12–E18, $p = 0.014$, differences for lesser intervals are not significant). On the other hand the proportion of insulin-positive cells, as a fraction of virus-transduced cells, is low for early and late stages and peaks for E14-initiated cultures at 29.7 ± 2.6%. By E18-initiated cultures it has fallen to 3.2 ± 0.09%. For the comparison between E14 and